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Abstract

Methods of identifying an acetyltransferase substrate in a sample are provided. Also provided are methods of localizing acetylation of an acetyltransferase substrate in a cell. Additionally, methods of labeling a protein substrate of an acetyltransferase are provided, as are methods of assaying an acetyltransferase in a sample. Methods of quantifying acetyltransferase activity in a sample are further provided. Compositions provided include halo-acetyl-pantetheine, halo-acetyl-pantetheine with a label, and halo-acetyl-CoA with a label on the adenine of the CoA.

What is claimed is:

1. A method of identifying an acetyltransferase substrate in a sample, the method comprising combining the sample with a labeled reagent and an acetyltransferase under conditions suitable for acetyltransferase enzyme activity, then identifying a substrate that has formed a base-stable covalent bond to the reagent,

wherein the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

2. The method of claim 1, wherein the acetyltransferase is a procaryotic acetyltransferase.

3. The method of claim 1, wherein the acetyltransferase is a eucaryotic acetyltransferase.

4. The method of claim 1, wherein the acetyltransferase is an archaeal acetyltransferase.

5. The method of claim 1, wherein the acetyltransferase is selected from the group consisting of a histone acetyltransferase, an N-terminal acetyltransferase, an arylamine N-acetyltransferase, an aminoglycoside acetyltransferase, chloramphenicol acetyltransferase, choline acetyltransferase, carnitine acetyltransferase, spermine acetyltransferase, and ornithine acetyltransferase.

6. The method of claim 1, wherein the reagent is a halo-acetyl-CoA.

7. The method of claim 6, wherein the halo-acetyl-CoA is a chloroacetyl-CoA.

8. The method of claim 6, wherein the halo-acetyl-CoA is a fluoroacetyl-CoA, a bromoacetyl-CoA, or an iodoacetyl-CoA.

9. The method of claim 6, wherein the halo-acetyl-CoA is labeled on the adenine group of the CoA.

10. The method of claim 1, wherein the label is radioactive.

11. The method of claim 10, wherein the radioactive label is ^{32}P.

12. The method of claim 1, wherein the label is fluorescent.

13. The method of claim 1, wherein the label is an affinity label.

14. The method of claim 13, wherein the affinity label is biotin.

15. The method of claim 1, wherein the substrate is a protein.

16. The method of claim 1, wherein the substrate is an antibiotic.

17. The method of claim 1, wherein the substrate is a metabolite less than 500 molecular weight.

18. The method of claim 1, wherein the sample comprises an extract of a cell.

19. The method of claim 1, wherein the substrate is identified by methods comprising gel electrophoresis.

20. The method of claim 1, wherein the substrate is identified by methods comprising mass spectroscopy and/or nuclear magnetic resonance.

21. A method of identifying an acetyltransferase substrate in a sample, the method comprising combining the sample with a reagent and an acetyltransferase under conditions suitable for acetyltransferase enzyme activity, then identifying a substrate that is associated with the acetyltransferase,

wherein the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine, and

wherein the acetyltransferase further comprises an affinity tag.

22. The method of claim 21, wherein the affinity tag is a his-6 tag.

23. A method of localizing acetylation of an acetyltransferase substrate in a cell, the method comprising combining the cell with a labeled reagent under conditions suitable for acetyltransferase enzyme activity, then determining the location of the label in the cell,

wherein the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

24. The method of claim 23, wherein the label is radioactive.

25. The method of claim 24, wherein the radioactive label is ^{32}P.

26. The method of claim 23, wherein the label is fluorescent.

27. The method of claim 23, wherein the label is an affinity label.

28. The method of claim 27, wherein the affinity label is biotin.

29. The method of claim 23, wherein the substrate is a histone.

30. The method of claim 23, wherein the location of the label in the cell is determined by light microscopy, autoradiography, or fluorescence microscopy.

31. The method of claim 23, wherein the cell is a eucaryotic cell.

32. The method of claim 23, wherein the cell is a prokaryotic cell.

33. A method of labeling a substrate of an acetyltransferase, the method comprising combining the substrate with the acetyltransferase and a labeled reagent under conditions suitable for acetyltransferase enzyme activity, wherein the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

34. The method of claim 33, wherein the acetyltransferase is selected from the group consisting of a histone acetyltransferase, an N-terminal acetyltransferase, an arylamine N-acetyltransferase, an aminoglycoside acetyltransferase, chloramphenicol acetyltransferase, choline acetyltransferase, carnitine acetyltransferase, spermine acetyltransferase, and ornithine acetyltransferase.

35. The method of claim 33, wherein the reagent is a halo-acetyl-CoA.

36. The method of claim 35, wherein the halo-acetyl-CoA is a chloroacetyl-CoA.

37. The method of claim 35, wherein the halo-acetyl-CoA is a fluoroacetyl-CoA, a bromoacetyl-CoA, or an iodoacetyl-CoA.

38. The method of claim 36, wherein the halo-acetyl-CoA is labeled on the adenine group of the CoA.

39. The method of claim 32, wherein the label is radioactive.

40. The method of claim 36, wherein the radioactive label is ^{32}P.

41. The method of claim 32, wherein the label is fluorescent.

42. The method of claim 32, wherein the label is an affinity label.

43. The method of claim 40, wherein the affinity label is biotin.

44. The method of claim 32, wherein the substrate is in a cellular extract.

45. A method of assaying an acetyltransferase in a sample, the method comprising combining the sample with a labeled reagent and an acetyltransferase substrate under conditions suitable for acetyltransferase enzyme activity, then determining whether the substrate that has formed a base-stable covalent bond to the reagent,

wherein the presence of the base-stable bond of the reagent to the substrate indicates the presence of an acetyltransferase in the sample, and

wherein the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

46. The method of claim 45, wherein the sample comprises an extract of a cell.

47. The method of claim 46, wherein the cell is a procaryotic cell.

48. The method of claim 46, wherein the cell is a eucaryotic cell.

49. The method of claim 45, wherein the reagent is a halo-acetyl-CoA.

50. The method of claim 49, wherein the halo-acetyl-CoA is a chloroacetyl-CoA.

51. The method of claim 49, wherein the halo-acetyl-CoA is a fluoroacetyl-CoA, a bromoacetyl-CoA, or an iodoacetyl-CoA.

52. The method of claim 49, wherein the halo-acetyl-CoA is labeled on the adenine group of the CoA.

53. The method of claim 45, wherein the label is radioactive.

54. The method of claim 53, wherein the radioactive label is ^{32}P.

55. The method of claim 45, wherein the label is fluorescent.

56. The method of claim 45, wherein the substrate is a protein.

57. The method of claim 56, wherein the protein is a histone.

58. The method of claim 45, wherein the substrate is an antibiotic.

59. The method of claim 45, wherein the substrate is a metabolite less than 500 molecular weight.

60. A method of quantifying acetyltransferase activity in a sample, the method comprising combining the sample with a labeled reagent and an acetyltransferase substrate under conditions suitable for acetyltransferase enzyme activity, then quantifying the labeled reagent that has formed a base-stable covalent bond to the substrate,

wherein the quantity of labeled reagent that has formed a base-stable covalent bond to the substrate is proportional to the acetyltransferase activity in the sample, and

wherein the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

61. The method of claim 60, wherein the sample comprises an extract of a cell.

62. The method of claim 61, wherein the cell is a procaryotic cell.

63. The method of claim 61, wherein the cell is a eucaryotic cell.

64. The method of claim 60, wherein the reagent is a halo-acetyl-CoA.

65. The method of claim 64, wherein the halo-acetyl-CoA is a chloroacetyl-CoA.

66. The method of claim 64, wherein the halo-acetyl-CoA is a fluoroacetyl-CoA, a bromoacetyl-CoA, or an iodoacetyl-CoA.

67. The method of claim 64, wherein the halo-acetyl-CoA is labeled on the adenine group of the CoA.

68. The method of claim 60, wherein the label is radioactive.

69. The method of claim 68, wherein the radioactive label is ^{32}P.

70. The method of claim 60, wherein the label is fluorescent.

71. The method of claim 60, wherein the substrate is a protein.

72. The method of claim 71, wherein the protein is a histone.

73. The method of claim 60, wherein the substrate is an antibiotic.

74. The method of claim 60, wherein the substrate is a metabolite less than 500 molecular weight.

75. A halo-acetyl-pantetheine.

76. The halo-acetyl-pantetheine of claim 75, wherein the halo group is a chloro-.

77. The halo-acetyl-pantetheine of claim 75, wherein the halo group is a fluoro-, a bromo-, or an iodo-.

78. A halo-acetyl-pantetheine with a label, wherein the label is a detectable label or an affinity label.

79. The halo-acetyl-pantetheine of claim 78, wherein the label is a radioactive label.

80. The halo-acetyl-pantetheine of claim 79, wherein the radioactive label is ^{32}P or ^{14}C.

81. The halo-acetyl-pantetheine of claim 78, wherein the label is a fluorescent label.

82. The halo-acetyl-pantetheine of claim 78, wherein the label is biotin.

83. A halo-acetyl-CoA labeled with ^{32}P, a fluorescent label, or an affinity label.

84. The halo-acetyl-CoA of claim 83, wherein the halo group is a chloro-.

85. The halo-acetyl-CoA of claim 83, wherein the halo group is a fluoro-, a bromo-, or an iodo-.

86. The halo-acetyl-CoA of claim 83, wherein the label is biotin.

87. A halo-acetyl-CoA with a label on the adenine of the CoA, wherein the label is a detectable label or an affinity label.

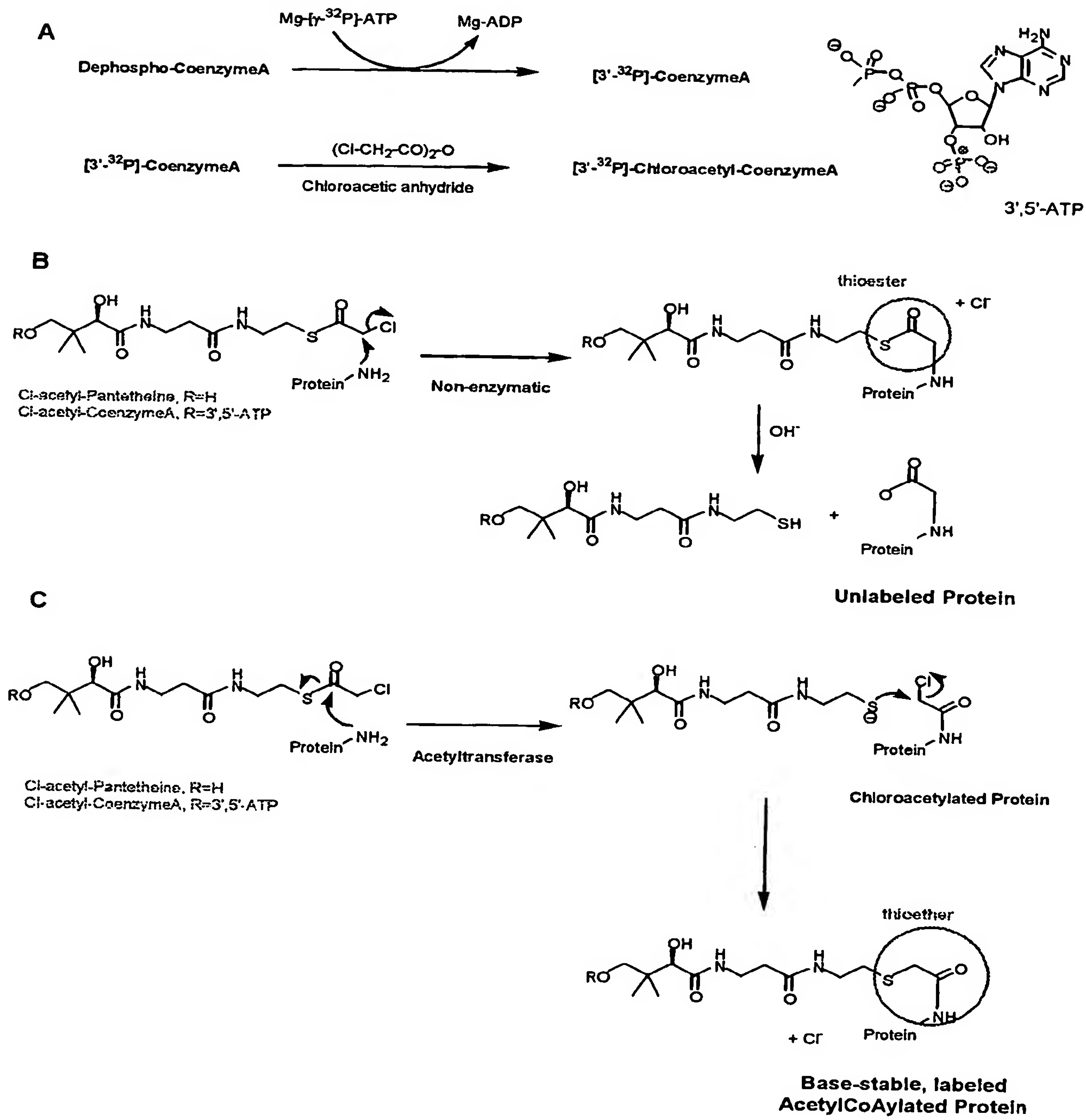
88. The halo-acetyl-CoA of claim 87, wherein the label is a radioactive label.

89. The halo-acetyl-CoA of claim 88, wherein the radioactive label is ^{32}P.

90. The halo-acetyl-CoA of claim 87, wherein the label is a fluorescent label.

91. The halo-acetyl-CoA of claim 87, wherein the label is biotin.

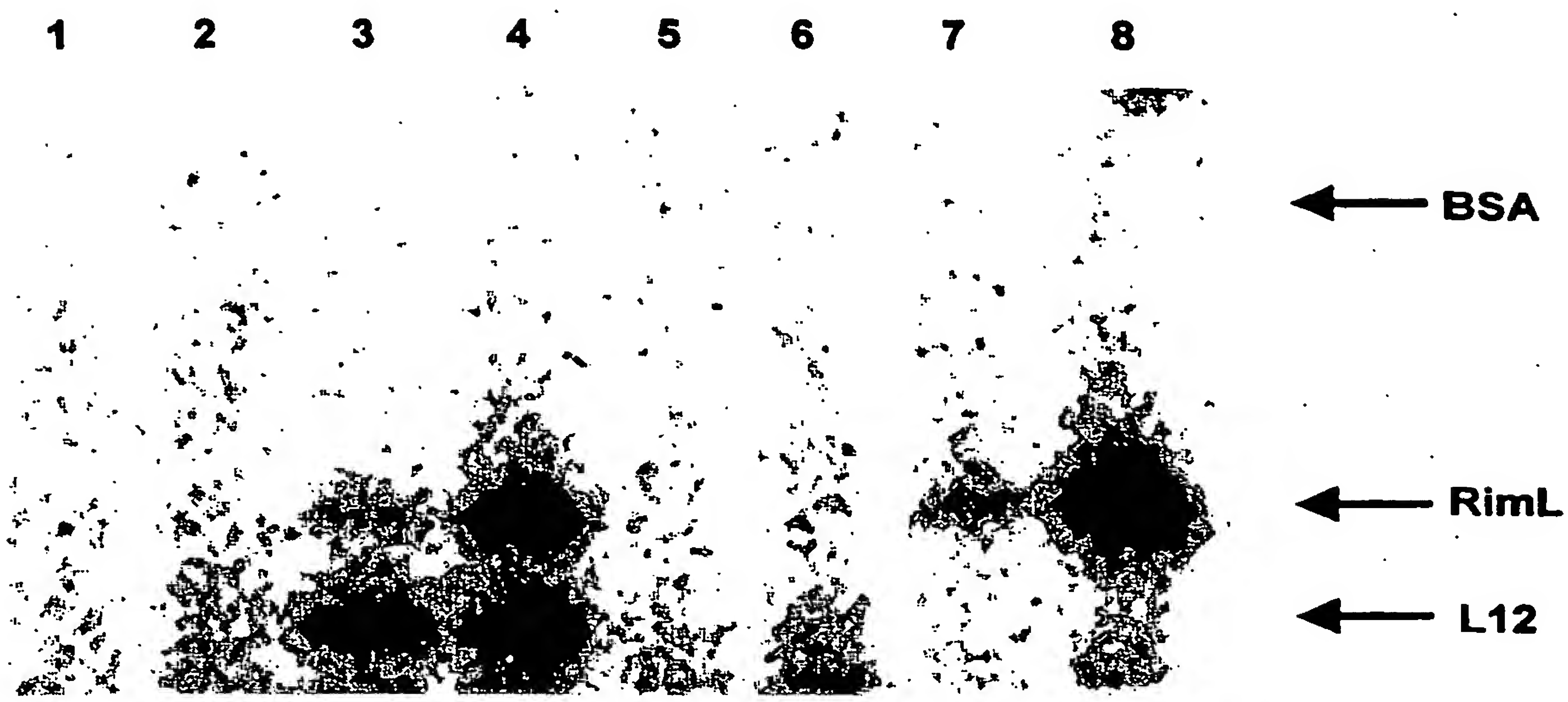
FIG. 1



A
Mg-[γ-^{32}P]-ATP
Mg-ADP
Dephospho-CoenzymeA
[3'-^{32}P]-CoenzymeA
[3'-^{32}P]-CoenzymeA
(Cl-CH$_2$-CO)$_2$-O
Chloroacetic anhydride
[3'-^{32}P]-Chloroacetyl-CoenzymeA
3',5'-ATP
B
thioester
Cl-acetyl-Pantetheine, R=H
Cl-acetyl-CoenzymeA, R=3',5'-ATP
Protein
Non-enzymatic
OH$^-$
Unlabeled Protein
C
Cl-acetyl-Pantetheine, R=H
Cl-acetyl-CoenzymeA, R=3',5'-ATP
Acetyltransferase
Chloroacetylated Protein
thioether
Base-stable, labeled
AcetylCoAylated Protein

FIG. 2

Labeling of L12 by RimL and [3'-^{32}P]chloro-acetyl-CoA



	1	2	3	4	5	6	7	8
BSA	+	+	-	-	-	-	-	-
RimL	-	-	+	+	-	-	+	+
L12	-	-	+	+	+	+	-	-
OH-	+	-	+	-	+	-	+	-

05/12/03

AR&E Docket No. 96700/820

New U.S. Provisional Patent Application (Small Entity Status)

Title: ASSAY FOR ACETYLTRANSFERASES AND ACETYLTRANSFERASE SUBSTRATES

Inventor: John S. Blanchard

Express Mail EV 335774548 US

251327.1

AR&E Docket No. 96700/820

Assay for Acetyltransferases and Acetyltransferase Substrates

Statement Regarding Federally Funded Research or Development

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided by the terms of Grant No. A133696 awarded by the National Institutes of Health.

Background

(1) Field of the Invention

The present invention generally relates to enzyme assays. More specifically, the invention is directed to novel assays for acetyltransferases and acetyltransferase substrates.

(2) Description of the Related Art

References cited

Polevoda and Sherman, 2003, J. Mol. Biol. 325:595-622.

Roth et al., 2001, Annu. Rev. Biochem. 70:81-120.

A rapidly developing area of cell signaling and regulation is the N-acetylation of proteins. These reactions are important in lifespan, transcriptional regulation, protein stability and drug resistance. Although rapid progress has been made in certain areas, the complete complement of substrates or targets for both procaryotic and eucaryotic acetyltransferases is presently unknown. In particular, bacterial genomes contain many open reading frames identified as members of the GNAT family of N-acetyltransferases (between 18 and 60, depending on the organism).

Acetyltransferases are enzymes that catalyze the transfer of the acetyl moiety from acetyl-CoA to their cognate substrates. These can be small molecule metabolites, antibiotics or proteins. The reaction mechanism can occur

in two different ways: the direct transfer of the acetyl group from acetyl-CoA to the substrate, or initial transfer of the acetyl group from acetyl-CoA to an enzyme group on the acetyltransferase to generate the acetyl-enzyme, and subsequent transfer of the acetyl group from the acetyl-enzyme to the substrate. Both of these mechanisms have been demonstrated.

Acetyltransferases are enzymes that catalyze the transfer of the acetyl moiety from acetyl-CoA to their cognate substrates. These can be small molecule metabolites, antibiotics or proteins. The reaction mechanism can occur in two different ways: the direct transfer of the acetyl group from acetyl-CoA to the substrate, or initial transfer of the acetyl group from acetyl-CoA to an enzyme group on the acetyltransferase to generate the acetyl-enzyme, and subsequent transfer of the acetyl group from the acetyl-enzyme to the substrate. Both of these mechanisms have been demonstrated.

The N-acetylation of proteins by acetyltransferases is a rapidly developing area of cell signaling and regulation. These reactions are important in lifespan, transcriptional regulation, protein stability and drug resistance. Although rapid progress has been made in certain areas, the complete complement of substrates or targets for both procaryotic and eucaryotic acetyltransferases is presently unknown. In particular, bacterial genomes contain many open reading frames identified as members of the GNAT family of N-acetyltransferases (between 18 and 60, depending on the organism).

Improved methods for isolating and identifying acetyltransferases and acetyltransferase substrates are thus needed. The present invention satisfies that need.

Summary of the Invention

Accordingly, the inventor has discovered that acetyltransferase substrates and acetyltransferases can be identified by combining the acetyltransferase and substrate with a halo-acetyl-CoA or halo-acetyl-pantetheine. The halo-acetyl-

CoA or halo-acetyl-pantetheine reacts with the acetyltransferase and substrate to form an acetyl-CoAylated substrate.

Thus, in some embodiments, the invention is directed to methods of identifying an acetyltransferase substrate in a sample. The methods comprise combining the sample with a labeled reagent and an acetyltransferase under conditions suitable for acetyltransferase enzyme activity, then identifying a substrate that has formed a base-stable covalent bond to the reagent. In these methods, the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

In other embodiments, the invention is directed to other methods of identifying an acetyltransferase substrate in a sample. The methods comprise combining the sample with a reagent and an acetyltransferase under conditions suitable for acetyltransferase enzyme activity, then identifying a substrate that is associated with the acetyltransferase. In these embodiments, the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine, and the acetyltransferase further comprises an affinity tag.

Additionally, the invention is directed to methods of localizing acetylation of an acetyltransferase substrate in a cell. The methods comprise combining the cell with a labeled reagent under conditions suitable for acetyltransferase enzyme activity, then determining the location of the label in the cell. In these methods, the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

The invention is also directed to methods of labeling a protein substrate of an acetyltransferase. The methods comprise combining the substrate with the acetyltransferase and a labeled reagent under conditions suitable for acetyltransferase enzyme activity. The reagent in these methods is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

In further embodiments, the invention is directed to methods of assaying an acetyltransferase in a sample. The methods comprise combining the sample with a labeled reagent and an acetyltransferase substrate under conditions suitable for acetyltransferase enzyme activity, then determining whether the substrate that has formed a base-stable covalent bond to the reagent. The

presence of the base-stable bond of the reagent to the substrate indicates the presence of an acetyltransferase in the sample. As in previous methods, the reagent in these methods is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

In related embodiments, the invention is also directed to methods of quantifying acetyltransferase activity in a sample. The methods comprise combining the sample with a labeled reagent and an acetyltransferase substrate under conditions suitable for acetyltransferase enzyme activity, then quantifying the labeled reagent that has formed a base-stable covalent bond to the substrate. The quantity of labeled reagent that has formed a base-stable covalent bond to the substrate is proportional to the acetyltransferase activity in the sample. In these methods, the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

The invention is further directed to halo-acetyl-pantetheine, as well as a halo-acetyl-pantetheine with a label, where the label is a detectable label or an affinity label, and a halo-acetyl-CoA labeled with ^{32}P, a fluorescent label, or an affinity label.

In related embodiments, the invention is directed to a halo-acetyl-CoA with a label on the adenine of the CoA, where the label is a detectable label or an affinity label.

Brief Description of the Drawings

FIG. 1 provides schematics of some chemical reactions useful for the present invention. Panel A provides a reaction scheme for producing [3'-^{32}P]-chloroacetyl-CoA. Panel B shows the nonenzymatic reaction of a halo-acetyl-CoA or a halo-acetyl-pantetheine (where the halo moiety is exemplified as a chloro). As indicated, the product of the nonenzymatic reaction is base-labile, and can thus be distinguished from the enzymatic reaction exemplified in Panel C. Panel C shows an example of the acetyltransferase reaction with Cl-acetyl-pantetheine or Cl-acetyl-CoA useful in the present invention.

FIG. 2 is an autoradiograph of an SDS-PAGE gel of the products of the reactions the incubation of RimL acetyltransferase (RimL) and its L12 substrate

(L12) with [3'-^{32}P]chloro-acetyl-CoA, in some cases further treating the reaction products with a pH 13 buffer (OH-). The BSA treatments were controls. These results validate the reaction mechanisms provided in FIG. 1.

Detailed Description of the Invention

The present invention is based on the discovery that acetyl-CoA or acetyl-pantetheine that is halogenated on the acetyl methyl moiety ("halo-acetyl-CoA" and "halo-acetyl-pantetheine", respectively) is transferred by acetyltransferase to an acetyltransferase substrate in place of the acetyl moiety. This discovery enables novel methods of identifying, purifying and quantifying acetyltransferases and acetyltransferase substrates. Relevant reactions are provided in FIG. 1. Panel A provides a method of producing a ^{32}P-labeled chloroacetyl-CoA. Such a labeled reagent, when used in an acetyltransferase reaction, labels the substrate and is therefore useful in identifying, purifying or quantifying acetyltransferases or acetyltransferase substrates. Panel B shows the base-labile nonenzymatic product of a halo-acetyl-CoA or a halo-acetyl-pantetheine with an acetyl group of a protein or small molecule (See Example). Since this product is base-labile, this non-enzymatic reaction can be distinguished from the acetyltransferase-mediated conjugation of a halo-acetyl-CoA or a halo-acetyl-pantetheine with an acetyltransferase substrate, exemplified in Panel C of FIG. 1. As shown in that Panel, and without being bound by the particular mechanism shown, the mechanism is believed to proceed as follows. The acetyltransferase causes the initial transfer of the haloacetyl group (here, the chloroacetyl group) to the substrate. The close proximity of the nucleophilic thiol of the CoA results in the attack of the thiol of CoA on the carbon atom to which the halogen (here, chlorine) is bound. This chemistry is well precedented, since α-halo-ketones are known to react with nucleophiles. The attack on the α-halo-ketone releases the halogen (here, chlorine) ion and generates a stable thioether linkage, as well as a stable amide linkage generated in the first step. When the halo-acetyl-CoA is labeled, for

example with ^{32}P (as shown in FIG. 1), the acetyltransferase substrate will become labeled, creating a convenient tag for identifying or quantifying an acetyltransferase or an acetyltransferase substrate.

Accordingly, in some embodiments, the invention is directed to methods of identifying an acetyltransferase substrate in a sample. The methods comprise combining the sample with a labeled reagent and an acetyltransferase under conditions suitable for acetyltransferase enzyme activity, then identifying a substrate that has formed a base-stable covalent bond to the reagent. In these embodiments, the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

Since acetyltransferases are known to function using the same general method of transfer of the acetyl group to the substrate, these methods would be expected to be useful with any acetyltransferase, now known or later discovered, including any procaryotic, eucaryotic or archaeal acetyltransferases. Non-limiting examples include histone acetyltransferases such as Gcn5, PCAF, Hat1, Elp3, Hpa2, Esa1, MOF, Sas2, Sas3, MORF, Tip60, Hbo1, P300, CBP, TAFII250, TFIIIC, Nut1, ACTR, and SRC1 (Roth et al., 2001); N-terminal acetyltransferases such as NatA, NatB and NatC (Polevoda and Sherman, 2003); arylamine N-acetyltransferases such as NAT1 and NAT2; aminoglycoside acetyltransferases; chloramphenicol acetyltransferases; choline acetyltransferases; carnitine acetyltransferases; spermine acetyltransferases; and ornithine acetyltransferases. Any substrate of any acetyltransferase is also useful in the present inventions, including proteins, oligopeptides, antibiotics, or small organic molecules less than 500 molecular weight.

As used herein, “halo” includes a chlorine, bromine, fluorine or iodine substitution on the acetyl group methyl moiety of acetyl-CoA or acetyl-pantetheine (see FIG. 1). Additionally, “halo-acetyl-CoA” and a “halo-acetyl-pantetheine” are not narrowly limited to the single unsubstituted species of each form, but encompasses the unsubstituted halo-acetyl-CoA and halo-acetyl-pantetheine as well as any substituted halo-acetyl-CoA and halo-acetyl-pantetheine that would be understood by the skilled artisan to be capable of

conjugation to an acetyltransferase substrate by an acetyltransferase. Non-limiting examples of such substitutions include any molecule up to about 500 mw conjugated to the adenine group of the halo-acetyl-CoA. In the three-dimensional structure of bacterial and eucaryotic acetyltransferases, CoA is bound in a way that the adenine ring makes few interactions with acetyltransferase and is essentially completely solvent exposed. Therefore, essentially any substitution on the adenine group would not be expected to interfere with the enzymatic action.

In preferred embodiments, the reagent is a halo-acetyl-CoA, since acetyl-CoA is the natural acetyl group contributor in acetyltransferase action. Since the halo-acetyl-CoA is exemplified herein (see Example) as chloroacetyl-CoA, that reagent is particularly preferred. However, fluoroacetyl-CoA, bromoacetyl-CoA, and iodoacetyl-CoA would be expected to also be useful in these methods. Selection of the most useful halo-acetyl-CoA for any particular purpose could be made by the skilled artisan without undue experimentation.

In many embodiments, the halo-acetyl-CoA or halo-acetyl-pantetheine reagent preferably further comprises a detectable label or an affinity label, for example, to easily detect and purify the acetyltransferase substrates binding the reagent. The invention is not narrowly limited to any particular detectable or affinity label, and the skilled artisan could select and utilize an appropriate label for any particular purpose without undue experimentation. Nonlimiting examples of useful labels include radioactive labels (e.g., ^{32}P, ^{14}C, ^{3}H), fluorescent labels, and affinity labels such as biotin. Where the reagent is a halo-acetyl-CoA, a preferred label is ^{32}P (see FIG. 1 and Example for methods of synthesizing and utilizing ^{32}P-halo-acetyl-CoA).

The present methods are useful with any sample comprising an acetyltransferase substrate. It is envisioned that the methods would be most useful where the sample is an extract of a cell.

The identification of the substrate in these methods could be by any appropriate means, including, for example, gel electrophoresis (especially useful

when the substrate is a protein - See Example), mass spectroscopy or nuclear magnetic resonance, or combinations of these methods.

Acetyltransferases are known to have a very high steady-state affinity for acetyl-CoA. Since the present invention provides for the covalent binding of acetyl-CoA or acetyl-pantetheine to the acetyltransferase substrate, the acetyltransferase would be expected to bind to the acetyl-CoA or acetyl-pantetheine that is covalently bound to the substrate. This suggests methods of identifying an acetyltransferase substrate in a sample. The methods comprise combining the sample with a reagent and an acetyltransferase under conditions suitable for acetyltransferase enzyme activity, then identifying a substrate that is associated with the acetyltransferase. In these embodiments, the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine, and the acetyltransferase further comprises an affinity tag. The affinity tag is used to purify the acetyltransferase-acetyl-CoA-substrate complex. The substrate on the purified complex is then identified by standard methods.

Any affinity tag that can be used to purify the complex can be used. In preferred embodiments, the affinity tag is a his-6 tag, since that tag can be readily incorporated into an acetyltransferase by molecular biological methods. As is well-known, his-6 tagged proteins are easily purified with Ni columns.

Since a labeled halo-acetyl-CoA or halo-acetyl-pantetheine covalently binds to acetyltransferase substrates, the location of the labeled substrates can be used to determine the location of the acetyltransferase activity in the cell. Such methods are useful for determining, e.g., whether a histone acetyltransferase also acetylates proteins located outside the nucleus, etc. Thus, the invention is additionally directed to methods of localizing acetylation of an acetyltransferase substrate in a cell. The method comprises combining the cell with a labeled reagent under conditions suitable for acetyltransferase enzyme activity, then determining the location of the label in the cell. In these embodiments, the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

In these methods, the label is preferably a detectable label such as a radioactive label, e.g., ^{32}P, or a fluorescent label. The label can also be an affinity label such as biotin, which can be detected, e.g., using avidin-labeled detectable enzyme such as peroxidase or alkaline phosphatase, using well known methods.

The location of the label in the cell can be determined by any appropriate method, e.g., light microscopy, autoradiography, or fluorescence microscopy. The most appropriate method for any particular application can be selected without undue experimentation.

These methods could be used with any procaryotic, archaeal, or eucaryotic cell. It would be expected to be most useful for eucaryotic cells, since they are known to be more compartmentalized than procaryotic or archaeal cells, and would therefore be expected to show more prominent localization of acetyltransferase activity than archaeal or procaryotic cells.

Since the halo-acetyl-CoA and halo-acetyl-pantetheine reagents previously described covalently bind stably to acetyltransferase substrates, the reagents can be used to label acetyltransferase substrates. Thus, in additional embodiments, the invention is directed to methods of labeling a substrate of an acetyltransferase. The methods comprise combining the substrate with the acetyltransferase and a labeled reagent under conditions suitable for acetyltransferase enzyme activity. In these embodiments, the reagent is a halo-acetyl-CoA or a halo-acetyl-pantetheine. These methods could be used with any acetyltransferase substrate, although it would be expected to be most useful with protein acetyltransferase substrates.

As with previously described methods, these methods could be used with any acetyltransferase, including histone acetyltransferases, N-terminal acetyltransferases, arylamine N-acetyltransferases, aminoglycoside acetyltransferases, chloramphenicol acetyltransferases, choline acetyltransferases, carnitine acetyltransferases, spermine acetyltransferases, and ornithine acetyltransferases. Also as with previously described methods, the

reagent is preferably a halo-acetyl-CoA, i.e., a chloroacetyl-CoA, a fluoroacetyl-CoA, a bromoacetyl-CoA, or an iodoacetyl-CoA. The label can also be on any moiety of the reagent that does not destroy its ability to bind to the substrate, for example on the adenine group of the CoA, as previously described. In preferred embodiments, the label is radioactive, e.g., ^{32}P, fluorescent, or an affinity label, e.g., biotin, but other labels can be used.

These methods could be used with a purified substrate and acetyltransferase, or with either or both in an impure state, e.g., in a cellular extract, a food, or an environmental sample.

The present invention is also directed to methods of assaying an acetyltransferase in a sample. The methods comprise combining the sample with a labeled reagent and an acetyltransferase substrate under conditions suitable for acetyltransferase enzyme activity, then determining whether the substrate that has formed a base-stable covalent bond to the reagent, where the presence of the base-stable bond of the reagent to the substrate indicates the presence of an acetyltransferase in the sample. As with the methods previously described, the reagent in these methods is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

These methods are not limited to any particular type of sample, and could include, for example, a food or an environmental sample. It would be expected that these methods would be particularly useful where the sample is an extract of a cell. Cellular extracts include those from archaeal, procaryotic, or eucaryotic cells.

As with methods described above, the reagent is preferably a halo-acetyl-CoA, i.e., a chloroacetyl-CoA, a fluoroacetyl-CoA, a bromoacetyl-CoA, or an iodoacetyl-CoA. Also as with methods described above, the label can also be on any moiety of the reagent that does not destroy its ability to bind to the substrate, for example on the adenine group of the CoA, as previously described. In preferred embodiments, the label is radioactive, e.g., ^{32}P, fluorescent, or an affinity label, e.g., biotin, but other labels can be used.

These methods are useful with any acetyltransferase substrate, including proteins, e.g., histones, antibiotics, and substrates that are metabolites less than 500 molecular weight.

The present invention is also directed to methods of quantifying acetyltransferase activity in a sample. The methods comprise combining the sample with a labeled reagent and an acetyltransferase substrate under conditions suitable for acetyltransferase enzyme activity, then quantifying the labeled reagent that has formed a base-stable covalent bond to the substrate. In these methods, the quantity of labeled reagent that has formed a base-stable covalent bond to the substrate is proportional to the acetyltransferase activity in the sample. As in methods described above, the substrate in these methods is a halo-acetyl-CoA or a halo-acetyl-pantetheine.

These methods are not limited to any particular type of sample, and could include, for example, a food or an environmental sample. It would be expected that these methods would be particularly useful where the sample is an extract of a cell. Cellular extracts include those from archaeal, procaryotic, or eucaryotic cells.

As with methods described above, the reagent is preferably a halo-acetyl-CoA, i.e., a chloroacetyl-CoA, a fluoroacetyl-CoA, a bromoacetyl-CoA, or an iodoacetyl-CoA. Also as with methods described above, the label can also be on any moiety of the reagent that does not destroy its ability to bind to the substrate, for example on the adenine group of the CoA, as previously described. In preferred embodiments, the label is radioactive, e.g., ^{32}P, fluorescent, or an affinity label, e.g., biotin, but other labels can be used.

These methods are useful with any acetyltransferase substrate, including proteins, e.g., histones, antibiotics, and substrates that are metabolites less than 500 molecular weight.

The skilled artisan could quantify the labeled reagent without undue experimentation using known methods for quantifying any label that would be utilized.

In additional embodiments, the present invention is directed to a halo-acetyl-pantetheine, which is novel, and rendered useful by the invention methods described above. The halo group of the invention halo-acetyl-pantetheine can be a chloro-, fluoro-, bromo-, or iodo- group.

In related embodiments, the invention is directed to a halo-acetyl-pantetheine as described immediately above, where the halo-acetyl-pantetheine has a detectable label or an affinity label. Nonlimiting examples of the label include radioactive labels, e.g., ^{32}P or ^{14}C, a fluorescent label, or the affinity label biotin.

In other related embodiments, the invention is directed to a halo-acetyl-CoA labeled with ^{32}P, a fluorescent label, or an affinity label (e.g., biotin). These compounds were previously unknown, and are made useful by the methods described above. These compounds encompass any halo group, i.e., chloro-, fluoro-, bromo-, or iodo-.

The invention is additionally directed to a halo-acetyl-CoA with a label on the adenine of the CoA, where the label is a detectable label or an affinity label. As with the other invention compounds described above, these compounds were previously unknown and are made useful by the novel invention methods. The detectable or affinity labels include those that have been previously described, e.g., radioactive labels (for example ^{32}P, ^{14}C, or ^{3}H), any known fluorescent label, or any affinity label, e.g., biotin.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

Example. An Assay for Acetyltransferases and Acetyltransferase Substrates

This Example provides some methods that can be used to assay for acetyltransferases and acetyltransferase substrates.

There are only three bacterial acetyltransferases whose substrates are known to be bacterial proteins. These are the *rimI*-, *rimJ*- and *rimL*- encoded a-N-acetyltransferases whose physiological substrates are the ribosomal proteins S5, S18 and L12. These three ribosomal proteins are post-translationally modified by the removal of the initiating formyl-methionine amino acid, and subsequently α-N-acetylated on their N-terminal alanine or serine residues. We have cloned, expressed and purified both the *S. enterica* RimL acetyltransferase and the L12 substrate. RimL is isolated in a highly a-N-acetylated form (>95%) while L12 is purified as a mixture of a-N-acetylated and non-acetylated forms (~25% acetylated, 75% non-acetylated).

Materials and Methods

Synthesis of [3'-^{32}P]Chloroacetyl-CoenzymeA. 60 μCi of [γ-^{32}P]-ATP was added to 1 μmole dephosphoCoA, 10 μmoles $MgCl_2$ and 3 nmoles of recombinant E. coli dephosphoCoA kinase in 50 mM Tris, pH 7.5. After ten min at 25 °C, 0.3 μmole of ATP was added. After an additional 20 minutes at 25 °C degrees, 1.5 μmoles of phosphoenolpyruvate, 8 units of pyruvate kinase and 2 more nmoles of dephosphoCoA kinase were added. The reaction mixture was incubated an additional 60 min at 37 °C. Enzymes were removed by passage through an Amicon 10 kilodalton molecular weight cutoff centrifugal filter, and the solution was lyophilized. The lyophilized powder was dissolved in 0.5 ml of 30 mM Tris buffer, pH 7.8 in 50% aqueous tetrahydrofuran. 0.2 ml of a solution containing 1.7 μmoles of chloroacetic acid and 2.3 μmoles of carbonyldiimidazole in tetrahydrofuran was added. The reaction was allowed to proceed for 2 h at room temperature, at which time, the thiol of CoA could not be detected by reaction with DTNB. This mixture was lyophilized.

Reaction of RimL and L12 with Chloroacetyl CoenzymeA. Four tubes were prepared that contained 1) 4 μM Salmonella enterica RimL, 2) 10 μM Salmonella enterica L12, 3) 4 μM Salmonella enterica RimL and 10 μM Salmonella enterica L12 and 4) 3 μM bovine serum albumin (BSA). 10 μl of a solution containing 75 mM Tris, pH 7.3 and 3.6 nmoles of [3'-^{32}P]chloroacetylCoA (0.2 μCi) was added to each tube (80 μM final concentration), and allowed to stand at room temperature for 90 min. The contents of each tube were equally divided, and half of the reaction mixture was adjusted to pH 13 by the addition of 200 mM CHES buffer, pH 13.5. These mixtures were incubated at 100 °C for two min. The solution pH was readjusted to pH 7-8 by the addition of 1N HCl. SDS-PAGE loading buffer was added to 5 μl aliquots of both the untreated and treated samples, and heated at 100 °C for 2 min. One μl aliquots were analyzed on 10-15% acrylamide gels using the Pharmacia PhastSystem. The gel was stained with Coomassie Blue, destained and dried. The radioactivity was determined using a PhosphoImager system.

Results

The results of these experiments are provided in FIG. 2. Incubation of purified RimL acetyltransferase and its L12 substrate with [^{14}C-acetyl]-AcCoA results in labeling of L12. Incubation of these purified proteins with [3'-^{32}P]-ClAcCoA causes both RimL and L12 to become radiolabeled, as shown in the autoradiogram of the SDS-PAGE gel in FIG. 2 (Lane 4). However, pre-treatment of this sample with base (pH 12, 100 °C, 5 minutes) followed by SDS-PAGE results in the nearly quantitative removal of the label from RimL, but nearly quantitative retention of the label by L12 (Lane 3). The small amount of retained label on RimL is very likely to represent the self-α-N-acetylation of the small amount of non-acetylated RimL originally present, a reaction that we have previously demonstrated. No reaction was observed with either L12 alone or BSA. These results validate the proposed reactions provided in FIG. 1.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. **EV 335774548 US**

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INVENTOR(S)

Given Name (first and middle [if any]	Family Name or Surname	Residence (City and either State or Foreign Country)
John S.	Blanchard	Pelham, NY

Additional inventors are being named on the one page Additional Page Cover Sheet *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

ASSAY FOR ACETYLTRANSFERASES AND ACETYLTRANSFERASE SUBSTRATES

Direct all correspondence to: CORRESPONDENCE ADDRESS

☐ Customer Number:

OR

☑ Firm or Individual Name	ARNOLD, Craig J.; GENDLOFF, Elie H.; MILLER, Alan D.				
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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 25

☑ Drawing(s) *Number of Sheets* 2

☐ Application Date Sheet. See 37 CFR 1.76

☐ CD(s), Number

☑ Other (specify) specification cover page; cover letter; return receipt postcard

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☑ A check or money order is enclosed to cover the filing fees.

☑ The Director is herby authorized to charge additional fees or credit any overpayment to Deposit Account Number: 01-1785

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE Amount ($): **$80.00**

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

[Page 1 of 2]

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME Elie H. Gendloff

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Date October 3, 2003

REGISTRATION NO. 44,704
(if appropriate)
Docket Number: 96700/820

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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October 3, 2003

Via Express Mail

Mail Stop Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

"Express Mail" mailing label No. EV 335774548 US
Date of Deposit: October 3, 2003
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.
Name: Elie H. Gendloff
Signature: [signature]

Re: Rule 1.53(c) Provisional Patent Application Claiming Small Entity Status
Title: ASSAY FOR ACETYLTRANSFERASES AND ACETYLTRANSFERASE SUBSTRATES
Inventor: John S. Blanchard
Our File: 96700/820

Dear Sir:

Pursuant to 37 C.F.R. §1.53(c), enclosed please find the following documents for filing with the above-identified provisional patent application claiming small entity status in the name of John S. Blanchard, entitled ASSAY FOR ACETYLTRANSFERASES AND ACETYLTRANSFERASE SUBSTRATES, comprising the following:

1. Provisional Application For Patent Cover Sheet (Form PTO/SB/16) (1 page);

2. Provisional patent application, including: application cover page (1 page), specification (15 pages), claims (9 pages), abstract (1 page), and drawings (2 sheets);

3. Amster, Rothstein & Ebenstein check in the amount of $80.00 to cover the provisional application filing fee for small entity status; and

4. Return receipt postcard.

Please acknowledge receipt of the enclosed papers by stamping the enclosed postcard and returning the same to us.

Respectfully submitted,

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